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Analysis of Two-Dimensional Gel Electrophoresis Map of Methicillin-Resistant *Staphylococcus aureus* Treated with Acetone Extract from *Canarium odontophyllum* Miq. Leaves

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) infection is a global health concern that has caused severe health threats over the past decade. Leaves extract of *C. odontophyllum* has been proven previously as an anti MRSA agent. Proteomics provide a technique that used to analyze the differential of protein expression profile between untreated and treated MRSA with subinhibitory concentrations of acetone extract from *C. odontophyllum* leaves. This study aims to determine the optimum parameter for analysis of protein expression profile using two-dimension gels electrophoresis (2-DE) for MRSA protein after treatment with acetone extract from *C. odontophyllum* leaves. Comparison of the Protein Expression Profile (PEP) between the untreated and treated MRSA was analyzed using PDQuest software. The optimum condition for MRSA protein treated with acetone extract from *C. odontophyllum* leaves to produce the best resolution with greater spot distribution was as follows: 100 µg volume of MRSA protein that loaded after passive rehydration then was run until reaching 25 kVrhs during IEF using 17 cm IPG strip within ranges of pH 4 - 7. Analysis of protein expression from the 2-DE gel map shows that 9 protein spots up-regulated and 41 protein spots were down-regulated with more than 2-fold differences ($p < 0.05$). This preliminary study on the PEP of MRSA treated with acetone extract of *C. odontophyllum* leave may provide an insight into the antimicrobial mechan-

ism, which could lead to the identification of target protein for future novel therapeutic development against MRSA infections.

Keywords

Canarium odontophyllum, MRSA, 2-DE, IEF, SDS-PAGE Protein Expression Profile

1. Introduction

Infectious diseases have been a leading cause of death worldwide. The rapid emergence and spread of drug-resistant organisms such as MRSA have posed a significant public health burden. MRSA has a highly prevalent infection, with more than 50% reported cases in hospitals worldwide, especially Malta, Asia, North and South America [1].

MRSA is strain of *S. aureus* with the presence of the *mec*A gene that encodes PBP2a, which is a cause of resistance in all β-lactam antibiotics [2]. The resistance of methicillin in *S. aureus* strains was due to the expression of methicillin hydrolyzing β lactamase as well as the expression of an altered form of PBP2 that binds to methicillin with lower affinity and higher rates of methicillin release [3]. In Malaysia, most strains were resistant to ciprofloxacin (92.5%), clindamycin (78.8%), erythromycin (93.4%) and gentamicin (86.8%) [4]. The MRSA strains also have been reported resistant against penicillins, cephalosporins, combinations of monobactams and carpenems and combinations of beta-lactam/beta-lactamase inhibitor [5]. Some standard anti MRSA antibiotics such as rifampicin, fusidic acid and vancomycin were still remained effective in treating the infections. However, a shift towards higher vancomycin MIC values and the failure of vancomycin treatment in vancomycin susceptible MRSA reported in [6]. The annual prevalence of MRSA in Malaysia is estimated to be 30% to 40% of all *S. aureus* infections [4]. Older age and comorbidities (diabetes mellitus, hypertension and chronic kidney disease) identified as the risk factors for MRSA infections [7]. The MRSA strain is ivided into HA-MRSA and CA-MRSA. Usually, HA-MRSA infection was acquired from the healthcare center while CA-MRSA infections acquired through the community. However, both HA-MRSA and CA-MRSA were significant factors of severe infections such as endocarditis, pneumonia, toxic shock syndrome, osteomyelitis and bacteraemia with a high rate of morbidity and mortality [5]. The MRSA infection causes a public health and economic burden worldwide through the longer length of stay and higher in-hospital mortality rate, antibiotic medication costs and total hospitalization costs [8].

Plants have various bioactive molecules that used as plant defense mechanisms against many pathogens. So, the use of plant extract and phytochemicals could be a great significance in treatment to help curb the problem of these multi-drug resistant organisms. *Canarium odontophyllum* Miq. which also known as "dabai" or Borneo olive, can found in Sumatra, Philippines and Borneo (Sarawak,

Sabah and East Kalimantan). It belongs to the family of Burseraceae genus *Canarium* L. [9] [10]. In the Asia-Pacific region, about 20 species of Burseraceae are used as a treatment for skin infection and wounds healing [10]. *C. odontophyllum* has shown to have antibacterial [11], antifungal [12], antioxidants [13], anti-cholinesterase [14] and anti-cancer activity [15]. The leaves of *C. odontophyllum*, which are pinnate, spiral and stipulated contain bioactive molecules, namely tannins, flavonoids, terpenoids, saponins and phenolics [11] [16].

Proteomic is a study of protein which includes protein expression pattern analysis, biological function, protein structure, variations during post-translational modification of proteins and protein-protein interactions [17]. Proteomics is an approach for the identification of target protein in pathogenic microorganisms through protein expression profile. In microbiology, it can help to understand the effects of the drug through the expression of a specific protein, use as a biological benchmark to detect disease and therapeutic development. It also provides information that occurs within cells and organisms through the pathways and networks of proteins that indirectly provide information on the function of a protein [18] [19] [20] [21].

Two-dimensional gel electrophoresis is a powerful technique for proteomic studies. It is frequently used to fractionate, identify and quantify proteins when coupled with mass spectrometric identification [22]. It is a method for separating proteins in a sample into a two-dimensional pattern of spots in a gel by combines the techniques of isoelectric focusing (IEF) with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [22]. The first-dimension step, which is IEF, was used to separates proteins according to their isoelectric points (pI). The isoelectric point is the pH at which the protein has zero net charge. Next, the protein was separated according to their molecular weights through second-dimension step by SDS-PAGE [23]. The 2-DE will create a 2-DE gel map of protein expression profile that used to identify the differential of protein expression. This method provides the first step for further analysis of the differential regulated protein spots using mass spectrometry.

In our previous study, *C. odontophyllum* has proven to show anti-MRSA activity which exhibits concentration dependent manner [24]. However, no further research on the treatment of *C. odontophyllum* leaves on the proteome of MRSA, which indirectly explain its mechanism of action. Separation of proteins with 2-DE presents technical challenges involving protein precipitation, load limitations and streaking. In this study, we aim to determine the optimum parameter for 2-DE analysis and compare the expression of proteins in MRSA treated *C. odontophyllum* leave extract. This preliminary study can provide important information in the study of *C. odontophyllum* leave on the proteome of MRSA, which indirectly explain its mechanism of action through protein expression profile pattern. In this study, we optimized the 2-DE system concerning the following aspects: sample volumes, sampling loading methods, total voltage and pH range of IPG stripes.

2. Materials and Methods

2.1. Preparation of Plant Extract

The leaf of *C. odontophyllum* purchased from Kuching, Sarawak, with voucher specimen number UKMB 40052. The whole leaf was used to prepare the extract [11]. The stock solution was prepared by dissolving the acetone extract of *C. odontophyllum* leaf powder in absolute acetone to achieve the final concentration of 100 mg/ml. The solution was vortex until completely dissolved and stored at 4˚C. The working solution of 20 mg/ml was prepared fresh by calculating the dilution of the stock solution then sterilized using a membrane filter of 0.45 µm pore size.

2.2. Bacterial Strain

The bacterial strains used in this study are MRSA Mu50 clinical strains obtained from the HUKM Faculty of Medicine Microbiology Laboratory.

2.3. Protein Extract

MRSA Mu50 was subculture onto Mueller Hinton agar (MHA) and incubated at 37˚C for 24 hours. The isolates were incubated in 10 mL of Mueller Hinton broth at 37˚C for 24 hours. The turbidity of the culture of bacteria was adjusted visually to an Optical Density (OD) of 0.08 at a wavelength of 625 nm using the spectrophotometer, which corresponded to 108 CFU/mL bacteria. The sub-inhibitory concentration of acetone extract of *C. odontophyllum* leaves against MRSA Mu50 at 156.25 µg/mL was used as treatment [24]. The extraction only involves bacteria cellular proteins and exoproteins were not included in the study. The samples were incubated in an incubator shaking at 37˚C for 8 hours at 100 rpm in the presence or absence of the acetone extract of *C. odontophyllum* leaves with biological triplicates. Then, the sample was centrifuged at 2200 xg for 30 min at 4˚C. The supernatant discarded and the pellet was suspended in 10 mL phosphate buffer saline solution (PBS). The resulting solution was spin again at 2500 xg for 15 min at 25˚C. Next, the pellet was suspended in 1 mL PBS and was centrifuged at 9000 xg for another 15 min at 25˚C. The supernatant was discarded. The pellet was suspended in 1 ml lysis buffer that contains protease inhibitor for sonication using an ultrasonic sonicator at 30% amplitude, 1 min plus on and 1 min plus off inside the beaker that has ice. Finally, the samples were centrifuged again at 11,600 xg for 10 min at 4˚C. The protein was purified using acetone precipitation to reduce the contamination of the protein. Four times the sample volume of cold acetone (−20˚C) was added into the tube that contains protein sample then, were vortex and incubate for 60 min at −20˚C. The sample was centrifuged for 10 min at 13,000 - 15,000 *x*g then the supernatant was removed. This step were repeated twice to remove the interfering substance. The acetone was allowed to evaporate from the uncapped tube at room temperature for 30 min. Finally, 100 µl lysis buffer was added into the sample then, was vortex thoroughly to dissolve the protein pellet. Protein concentrations were quan-

tified by the Bradford protein assay kit (Bio-Rad) according to the manufacturer's instruction. Bovine serum albumin (BSA) used as a protein standard. The samples were stored at −20˚C until required.

2.4. 2-D Gel Electrophoresis

Isoelectric focusing (IEF) was performed with the Protean® IEF Cell. Dry strips initially rehydrated for 24 hours with 350 µL of rehydration buffer comprising 7M urea, 2M thiourea, 4% 3-[(cholamidopropyl) dimethyl ammonio]-1-propane sulfonate, 0.5% IPG buffer (pH 3 - 10), DTT and trace amounts of bromophenol blue. The difference sample loading method was applied to IPG DryStrips. 1) Passive rehydration using sample in-gel rehydration method: Sample protein was added into the rehydration buffer to allow the absorption of the sample during rehydration without the presence of an applied voltage. 2) Active rehydration: Sample protein was added into rehydration buffer to allow the absorption of sample during rehydration with presence of low voltage between 30 - 50 V. 3) Passive rehydration by paper-bridge sample loading method: Sample applied to a small piece of electrode strip paper then, was loaded at the cathode into a rehydrated IPG strip during run IEF. 4) Passive rehydration by introducing the sample shortly before running IEF.

The IPG strips focused at 50 µA for 25 kVhrs, 57 kVhrs and 87 kVhrs, respectively at 20˚C. The focused IPG strips were left to equilibrate for 15 min in equilibration buffer (6M urea, 75 mM Tris-HCl pH 8.8, 29% glycerol, 2% SDS and trace amounts of bromophenol blue) containing 1% DTT. The focused IPG strips alkylated in the standard sample buffer consisting of 2.5% iodoacetamide (IAA) for 15 min. The IPG strips of reduced and alkylated samples were transferred into SDS-PAGE gels and sealed with agarose gel for second-dimensional protein separation.

The second dimension was run using Biorad PROTEAN II xi cell at 80 V for 1 hour then the voltage was then increased to 500 V until the bromophenol blue tracking dye reached the bottom of the gel. This protocol of 2-DE were performed according to the previous method with technical triplicate [25].

2.5. Silver Staining

The silver staining was performed with some modification [26]. The reagent was prepared fresh before use. The 2-DE gel was fixed overnight in a mixture of ethanol and glacial acetic acid before sensitization process for 30 min then were washed three times with distilled water for 5 min. The gel was transfer into a silver reaction solution for 20 min then were rinsed twice for 1 min. Next, the gel was soaked in a developing process solution for 10 min before been transfer to the stopping reaction solution. The reaction process was run for 10 min before being rinsed with distilled water. The whole process was conducted using a shaker machine. The 2-DE gel can be preserved in a preservation solution for storage or directly analyzing using the software.

2.6. Image Scan Analysis

The gel images captured using the Molecular Imager GS-800 USB Calibrated Densitometer was analyzed using PDQuest Advance 2-D analysis software v8.0 (Bio-Rad). The statistic used for analysis in this study was Student's t-test in the PDQuest Advance 2-D analysis software. The software was used to compare the protein expression profile between the images of 2-DE gel treated with acetone extract of *C. odontophyllum* leaves with the untreated. The images of 2-DE gel protein were analyzed from three biological replicate and three technical replicates. The differential of protein expression profile was analyzed based on significant ($p < 0.05$) protein spots with more than 2-fold change in relative spot volume.

3. Results

3.1. Optimization of Two-Dimensional Gel Electrophoresis

The optimal protein concentration is an important factor in ensuring an exact resolution of 2-DE gel. The results on the comparative 2-DE gel of MRSA protein concentration at 50 µg, 60 µg, 80 µg, 100 µg, 120 µg and 150 µg shown in Figure 1. Based on Figure 1, 100 µg of protein sample loaded in IEF, yielded the best quality of resolution and produced more clearly defined spots compared to the protein concentration loaded at 50 µg, 60 µg, 80 µg, 120 µg and 150 µg. At protein concentration of 50 µg, 60 µg and 80 µg, the spots produced in the gel was fades and less while at a protein concentration of 120 µg and 150 µg, the 2-DE gel show more overlapping spot and a reduction in the number of spots compared to 100 µg.

A comparison of the sample loading method of optimum protein concentration in Figure 2 showed that passive rehydration by introducing the sample shortly before run IEF was the most suitable method by showing better reproducibility with a greater number of more apparent protein spots compared to active rehydration, sample in-gel rehydration and paper-bridge sample loading method. Active rehydration and sample in-gel rehydration visualize localized horizontal spot streaking. The paper-bridge sample loading method displayed an impressive spot resolution. However, there is a predominant loss of protein spot.

The comparison of difference in total voltage showed in Figure 3. Based on Figure 3, the total voltage of 25 kVhrs shows improvement in image quality resolution with less horizontal spot streaking compares to longer focusing time. The total voltage of 57 kVhrs and 87 kVhrs show distorted protein patterns, horizontal streaks and loss of proteins.

Figure 4 shows that the differential of the protein expression profile of MRSA treated with acetone extract of *C. odontophyllum* leave was more focused on pH 4 - 7 compared to pH 3 - 10. Most of the protein was located at range pH 4 - 7 and well distributed compare to pH 3 - 10 which, most of the protein shifted to the left and cause protein overlapping. The 2-DE gel map of pH 4 - 7 demonstrated not only improved resolution and higher spot numbers but also visualize low abundance proteins and unresolved spot clusters.

3.2. Analysis of Differential of Protein Expression Profile

The mapping of cellular protein expression in MRSA revealed distinct alterations between the two proteomes. A total of (828 ± 88.38) protein spots detected in protein expression profiles of MRSA treated with acetone extract of *C. odontophyllum* leave while (677 ± 96.96) protein spots detected in untreated MRSA. Figure 5 showed the intersection between quantitative analysis with significant spot protein of cellular protein expression for 50 protein spots. Based on Figure 6, Figure 7 protein spots up-regulated and 41 protein spots were down-regulated with more than twofold differences ($p < 0.05$) after treatment with the extract.

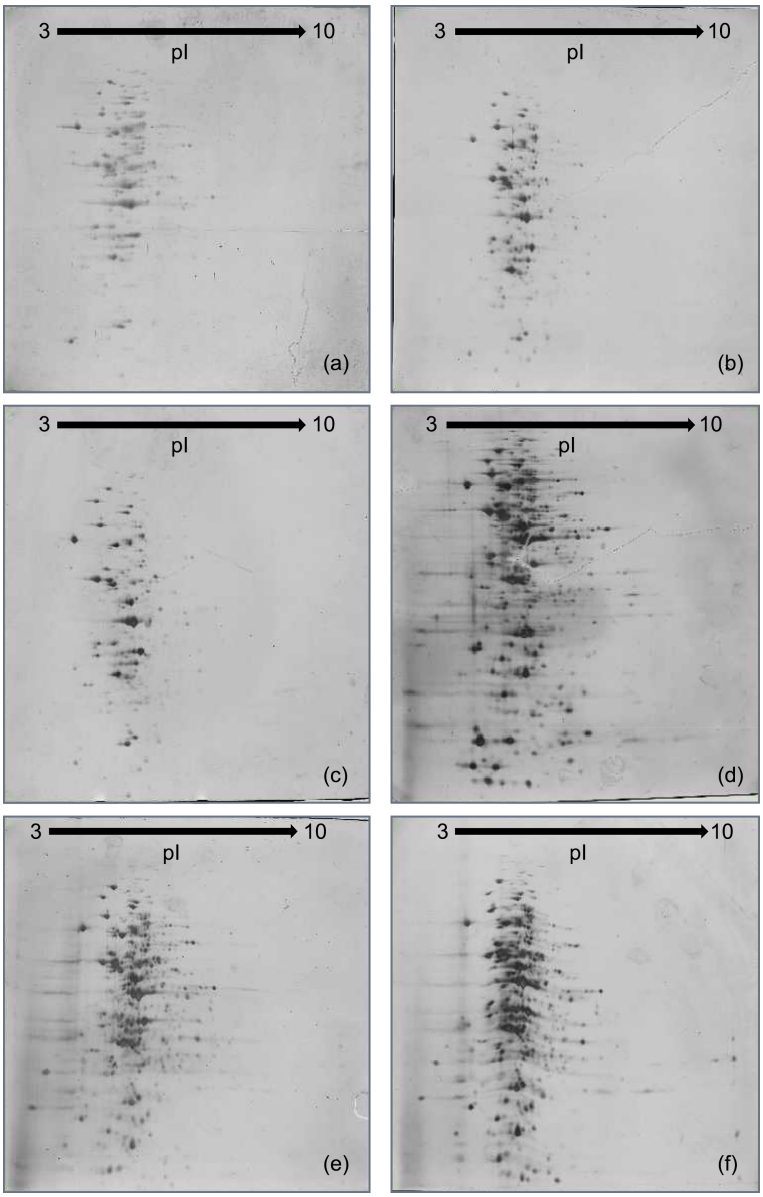

Figure 1. Comparison of the 2-DE gel protein spot resolution of MRSA Mu50 treated with acetone extract of *C. odontophyllum* leave at different concentrations using 17 cm IPG strips within the pH range 3 - 10. (a) 50 µg (b) 60 µg (c) 80 µg (d) 100 µg (e) 120 µg and (f) 150 µg.

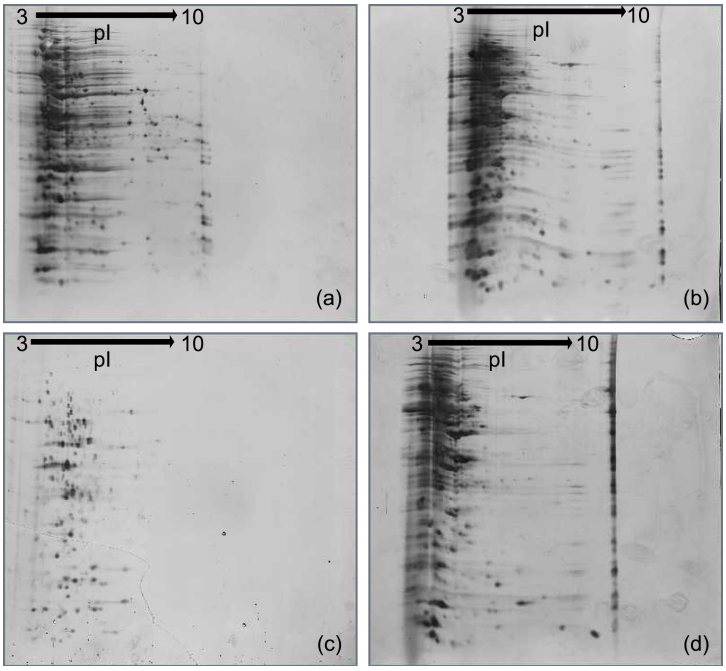

Figure 2. Comparison of the 2-DE gel protein spot resolution of MRSA Mu50 treated with acetone extract of *C. odontophyllum* leave by various sample loading method using 11 cm IPG strips pH range 3 - 10. (a) Passive rehydration method: Sample in-gel rehydration method (b) Active rehydration method (c) Passive rehydration method: Paper-bridge sample loading (d) Passive rehydration method: Samples were introduced shortly before IEF.

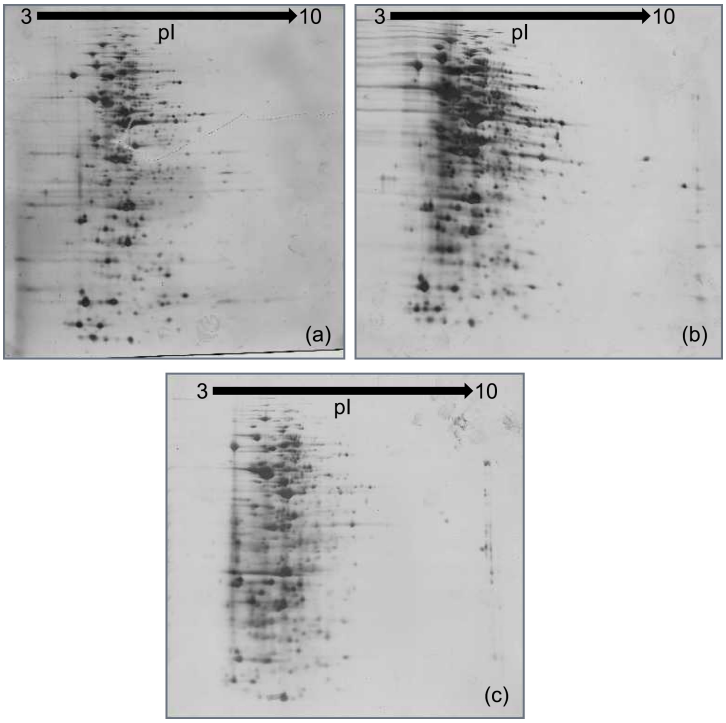

Figure 3. Comparison of the 2-DE gel protein spot resolution of MRSA Mu50 treated with acetone extract of *C. odontophyllum* leave using a 17 cm IPG strips at pH range 3 - 10 using a total voltage of (a) 25 kVhrs (b) 57 kVhrs (c) 87 kVhrs.

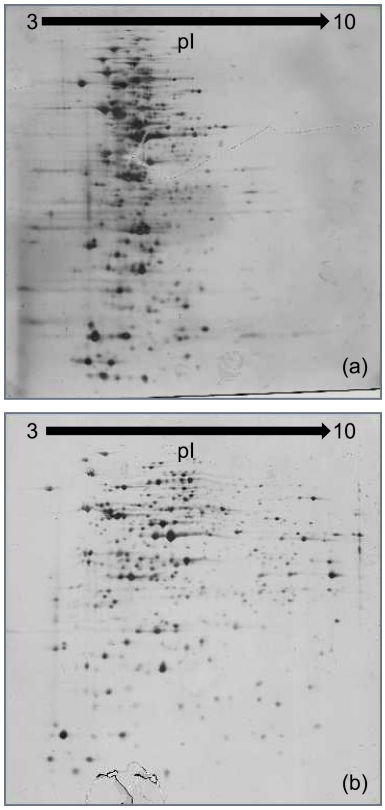

Figure 4. Comparison of the 2-DE gel protein spot resolution of MRSA Mu50 treated with acetone extract of *C. odontophyllum* leave using 17 cm IPG strips at different pH ranges. (a) pH 3 - 10 (b) pH 4 - 7.

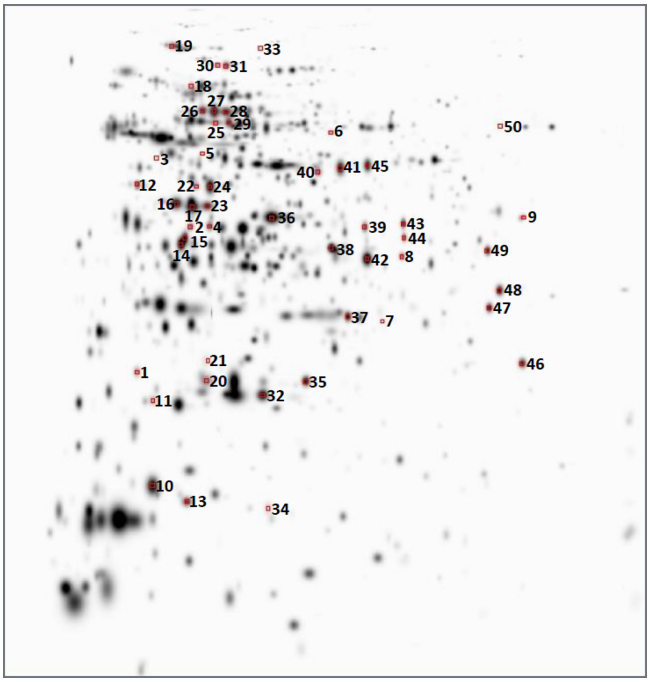

Figure 5. The intersection between quantitative analysis with significant spot protein of differential MRSA cellular protein expression of treated and untreated acetone extract of *C. odontophyllum* leave.

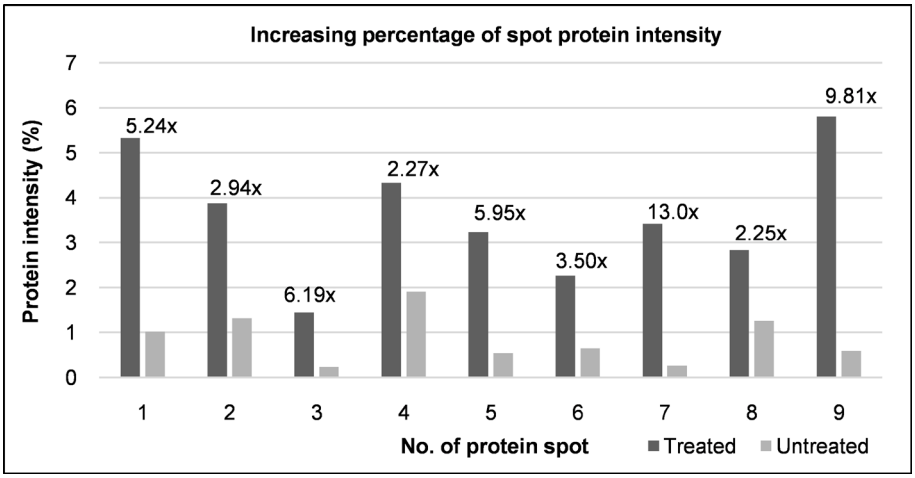

Figure 6. Histogram showing the increase in relative protein intensity (%) for the nine spots determined by PDQuest Advance 2-D analysis software v8.0. The increase in protein expression of MRSA Mu50 in the nine spots obtained by dividing the percentage of protein in the treated group by the untreated group.

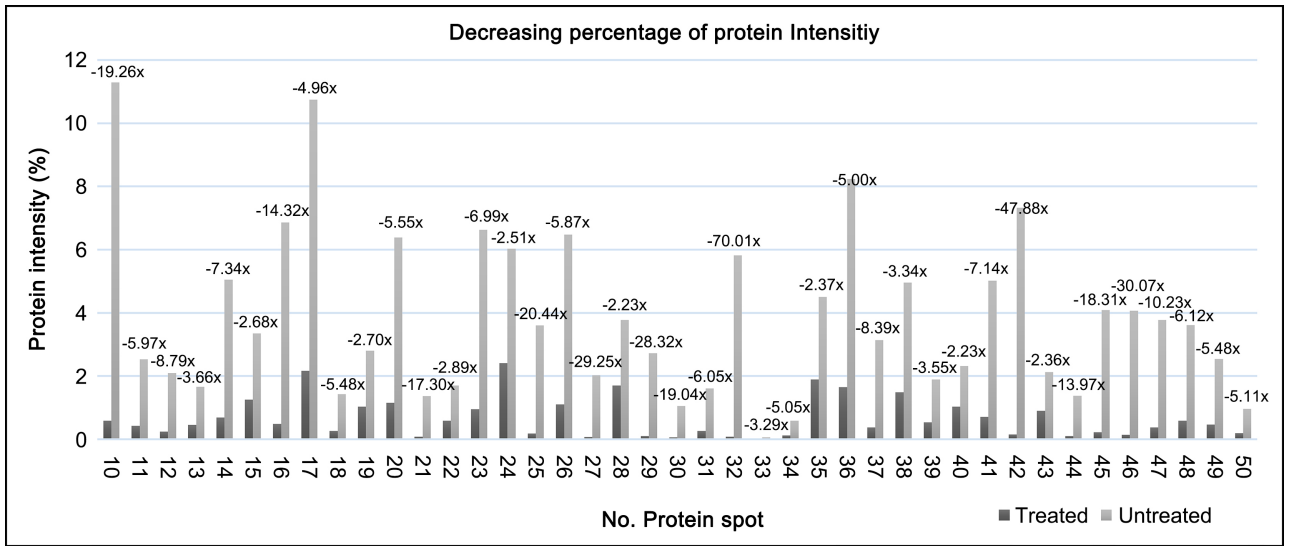

Figure 7. Histogram showing the decrease relative protein intensity (%) for the 41 spots determined by PDQuest Advance 2-D analysis software v8.0. The decrease in protein expression of MRSA Mu50 in the 41 spots obtained by dividing the percentage of protein in the treated group by the untreated group.

4. Discussion

The optimum conditions for each sample of 2-DE gel were different [27]. The protein needs to be optimized to choose the optimum condition for 2-DE to make sure the protein expression profile map in the best resolution and avoid false positive.

Proteins can bind to phenolic elements of the media through mechanisms such as hydrogen interactions, ionic and hydrophobic interactions to produce more hydrophobic products that facilitate protein aggregation and precipitation [28]. The acetone extract of *C. odontophyllum* leave contains phenolic elements that believed to disrupt the protein resolution of the gel and affect the back-

ground [29].

The protein from bacteria was prior extract. During the extraction process, the bacteria were treated with sub-inhibitory concentration, which is $\frac{1}{2}$ × MIC (156 μg/ml) of 8-hour incubation. During this state, the extract shows bacteriostatic activity toward MRSA, which the extract inhibits the growth of bacteria. The treatment time and concentration were choosing based on MIC and TKA from our previous study to ensure that enough protein can be obtained and minimize the loss of protein during the protein extraction process for protein expression profile analysis [30]. Decreasing of 50% - 70% of treated bacterial growth rate compared to untreated groups is useful for assessing antibiotic action against proteomic bacteria [31].

Based on the optimization of sample concentration, 100 μg shows the best resolution and more spot protein compare to others. The protein concentration of 50 μg, 60 μg and 80 μg show fades and less spot due to low protein load and cause undetected low abundance proteins. The protein concentration of 120 μg and 150 μg show more overlapping spot might be due to the overloading of sample protein. The low abundance proteins could only be analyzed if a larger starting concentration of proteins used [32]. The reduction in the number of spots at higher protein load was probably because the capacity of the gel to absorb protein is limited when the concentration of protein samples exceeded the solubility threshold of some proteins [33]. The solubilization of protein is very important to achieve good focusing and to get the best image of gel resolution [34]. The loss of proteins during rewelling increases as the amount of protein loaded amplified [35]. The absorption capacity of each gel sample protein varies depending on their molecular size [25].

Passive rehydration by introducing the sample shortly before run IEF was chosen as the optimal method in this study as it showed more protein spots compared to other sample rehydration application methods. The sample loading method in the IEF tray allows proteins that cannot be absorbed into the gel strip to extracted with low voltage assistance at the beginning of the IEF process [36]. Loss of protein during active rehydration may be due to the loss of small proteins with high mobility [37]. Besides, 50% protein loss can occur during passive rehydration if the sample of proteins is large [36]. Although the loading of the paper-bridge sample shows an impressive spot resolution, there is a predominant loss of protein spot [38]. Localized horizontal spot streaking may be due to improper IPG strip rehydration, which caused by unevenly distributed in the rehydration solution or absorption of impurity during rehydration [39].

Optimal focusing time important to improve the 2-DE gel image quality and reproducibility. The resolution of the 2-DE gel image was determined by the slope for the gradient pH and electric field strength. IEF performed in the denaturation phase provides high resolution and a clean image [40]. Inadequate IEF processes can induce horizontal and vertical distortion. At the same time, excessive IEF can cause distorted pattern proteins and horizontal streaking at the ends

of the gel base, causing protein loss [41]. Based on the optimization of total voltage for IEF, 25 kVhrs shows the best resolution with less horizontal streaking compare to 57 kVhrs and 87 kVhrs. This result proves that overfocusing does not result in the migration of proteins towards the cathode but will result in excess water exudation at the surface of the IPG strips due to active water transport, which leads to distorted protein patterns, horizontal streaks and loss of proteins [42].

During IEF, the protein will move along the pH gradient under the influence of the electric field and will focus on the location where the pH value is equal to pI, which is the pH where the protein net charge is zero. The choice of pH gradient depends on sample [42]. Based on the protein mapping on the 2-DE gel for the MRSA protein, it found that most of the protein spots concentrated in the acidic pH range. Differential expression of the treated MRSA protein with acetone extract of *C. odontophyllum* leave was focusing at pH 4 - 7 instead of pH 3 - 10. The 2-DE gel map using pH 4 - 7 well distributed compare to pH 3 - 10, which most of the protein was shifted to the left and resulting in an overlap of protein spots. Broad pH range commonly used to resolve all proteins, still resolution quality of this scale is low compared to narrow-scale pH gradients that showed an increase in the resolution and detection of low abundance proteins [43]. The narrow-scale pH gradient indicates an increase in the number of protein spots as it can separate the combined protein spots [44].

Analysis of differential protein expression profile between the treated and untreated MRSA proteins is a reference to show changes in the bacterial proteome in response to treatment agents to identify modes of action and treatment targets [45]. The 2-DE gel map shows a difference in the protein expression profile pattern between treated and untreated MRSA. A total of 9 protein spots show an increase in expression, while 41 protein spots had a significantly decreased in expression with more than a 2-fold difference. Each protein encoded by a gene has its role. Gene expression is a mechanism used by cells to increase or decrease the production of gene products in response to bacterial physiology of environmental change [46].

S. aureus RN1HG001 proteome treated with quinolonyl-oxazolidinone antibody MCB3681 showed 13 induced proteins while 16 suppressed proteins [47]. Suppression proteins are from different amino acid production sites, amino acid-tRNA synthetases and the resistance factor Methicillin protein FemB. The mechanism of action of juglone, naftokuinon from plants against *S. aureus* carried out using the 2-DE method, showed differential of 21 protein spots expression between treated and untreated cells. A total of 13 identified protein spots are involved in the inhibition of protein synthesis, tri-carboxylic acid cycle as well, as DNA and RNA synthesis [45].

5. Conclusion

In conclusion, the optimum condition to run 2-DE for MRSA protein treated

with acetone extract of *C. odontophyllum* leaves was passive rehydration by introducing 100 µg concentration of MRSA protein shortly before running IEF using 17 cm IPG strip pH 4 - 7 at a total voltage of 25 kVhrs. The differential of protein expression profile for MRSA treated with acetone extract from *C. odontophyllum* leaves showed up-regulation of 9 proteins and down-regulation of 41 proteins. This information has shed light on the antimicrobial mechanism, as an alternative for therapeutic agent to treat MRSA infection. However, this study alone is not enough to identify the protein. Therefore, a further study in target proteins identification using MALDI-TOF MS/M is needed.

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Declaration of Conflicting Interests

The authors report no conflicts of interest. The authors are responsible for the content and writing of this article.

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